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PAPER

Monoclonal antibody-based broad-specificity immunoassay for monitoring organophosphorus pesticides in environmental water samples

Zhen-Lin Xu,[a] Dao-Ping Zeng,[a] Jin-Yi Yang,[a] Yu-Dong Shen,[a] Ross C. Beier,[b] Hong-Tao Lei,[a] Hong Wang[a] and Yuan-Ming Sun*[a]

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The development of easy-to-use and rapid-monitoring immunoassay methods for organic environmental pollutants in a class-selective manner is a topic of considerable environmental interest. In this work, a heterologous competitive indirect enzyme-linked immunosorbent assay (ciELISA) based on a monoclonal antibody (MAb) with broad-specificity for organophosphorus pesticides (OPs) was applied to the detection of *O*,*O*-diethyl and *O*,*O*-dimethyl OPs in water samples. The ciELISA conditions were carefully optimized to obtain a three to five-fold improvement of sensitivity for most OPs, and thirteen OPs were determined at concentrations ranging from 0.017 to 30 ng mL^{-1}. The determination of spiked environmental water samples showed average recoveries from 81.5% to 115.1%, with the coefficient of variation (CV) ranging from 6.1% to 20.9%, which showed satisfactory reproducibility of the developed ciELISA. To overcome the negative aspect of broad-specificity immunoassays not providing qualitative and quantitative analysis of individual OPs in blind samples, we used "percent inhibition rate" to make the developed ciELISA a semi-quantitative method, which allows the monitoring of positive samples from hundreds of negative samples. The determination of OPs in blind water samples by the developed ELISA with confirmation by HPLC-MS/MS analysis demonstrated that the assay is ideally suited as a screening method for OP residues prior to chromatographic analysis.

1. Introduction

Today, organophosphorous pesticides (OPs) have become the most widely used pesticides in both agricultural and domestic settings, especially in developing countries.[1] Although they are degraded under many environmental conditions, extensive use of OPs has led to the contamination of surface waters by drift, runoff, drainage and leaching, which is a topic of considerable environmental interest.[2] Environmental monitoring studies are necessary to evaluate the risk to non-target species' health and to ensure that they are not being exposed to OPs. Instrumental methods such as gas chromatography-mass spectrometry (GC-MS) or high-performance liquid chromatography-MS (HPLC-MS) are often methods of choice for OP analyses. But they require complex and expensive instrumentation that has to be managed by highly qualified personnel, usually involve extensive purification and often requires derivatization of the target compound, and these methods are not conducive to large scale

[a]*Guangdong Provincial Key Laboratory of Food Quality and Safety, South China Agricultural University, Guangzhou, 510642, China. E-mail: ymsun@scau.edu.cn; Fax: +86-(20)-85280270; Tel: +86-(20)-85283925*
[b]*Food and Feed Safety Research Unit, Southern Plains Agricultural Research Center, Agricultural Research Service, U.S. Department of Agriculture, 2881 F&B Road, College Station, TX, 77845-4988, USA*

Environmental impact

The extensive use of organophosphorus pesticides (OPs) in agriculture and in other uses has resulted in extensive environmental OP residue contamination, especially in developing countries. Environmental monitoring studies are necessary to evaluate the health risk to non-target species. As an alternative to the laborious and expensive instrumental methods, we developed a monoclonal antibody-based broad-specificity ELISA that can determine more than ten OPs simultaneously within a short time period. The developed ELISA was applied to blind environmental samples as a semi-quantitative method, with simultaneous confirmation by HPLC-MS/MS. Our results indicate that the developed ELISA method had good accuracy and reproducibility, and was an effective monitoring test method for a broad-array of OPs that can be used prior to chromatographic analysis, thereby reducing the costs of labor and expensive instrumental analysis.

screening studies.[3,4] As an alternative, antibody-based immunoassays such as the enzyme-linked immunosorbent assay (ELISA) have proven to be rapid, sensitive and a cost effective analytical tool for routine monitoring.[5,6] Many immunoassay methods used for determination of pesticides have been approved and listed by the U.S. Environmental Protection Agency (EPA).[7]

Until now, most immunoassays available for the determination of OPs have been specific for only one single analyte. They are useful during screening analysis when a large number of samples have to be analyzed in parallel for a single analyte within a short period of time.[8] However, their specificity can be a limitation in some circumstances. For example, when screening a large number of samples that may potentially contain several different OP residues, there would be a need to develop several immunoassay methods, one for each OP. In cases like this, a broad-specificity immunoassay that can detect more than a single target in a class-selective manner would be beneficial.[9]

After the ban on the usage of highly toxic OPs such as methamidophos, dichlorvos and dimethoate, some alternative candidates such as coumaphos, phoxim, quinalphos, triazophos and azinphos-ethyl were widely used.[10–14] Since these OPs share one of the common moieties, *O*,*O*-diethyl phosphorothioate or phosphorothionothiolate, it is possible to prepare broad-specificity antibodies against the *O*,*O*-diethyl phosphorothioate or phosphorothionothiolate groups.[15–17] However, most developed assays lacked the required specificity or sensitivity. Moreover, one negative aspect of broad-specificity immunoassays is that they cannot provide qualitative and quantitative analysis of individual OPs in blind samples, which is due to their non-uniform cross-reactivity (CR) to different OPs. Therefore, the application of broad-specificity immunoassays to blind samples was not generally attempted.[15–17]

As an extension of our previous study,[18] this work describes the development of a monoclonal antibody (MAb)-based broad-specificity competitive indirect ELISA (ciELISA) for eighteen *O*,*O*-diethyl OPs and thirteen *O*,*O*-dimethyl OPs. The influence of several physicochemical factors such as ionic strength, pH, organic solvent and Tween® 20 concentrations were carefully evaluated for their effect on ciELISA performance. To apply the developed ciELISA[18] to analyze environmental water samples, we used "percent inhibition rate" to make the ciELISA a semi-quantitative method, which allowed the screening of positive samples from hundreds of negative samples. The positive samples were then confirmed by multi-analyte analysis with high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). Our results indicated that the broad-specificity ciELISA was a feasible and effective screening test for a broad-array of OPs prior to chromatographic analysis.

2. Experimental

2.1 Reagents and instruments

Analytical OP standards were purchased from Dr Ehrenstorfer GmbH (Augsburg, Germany). Immunogen and coating antigen (Fig. 1), as well as the monoclonal antibody were self-prepared as previously described.[18] 3,3′,5,5′-Tetramethylbenzidine (TMB) and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (HRP-IgG) were obtained from Sigma-Aldrich (St. Louis, MO, USA). *N*,*N*-Dimethylformamide (DMF), methanol, acetone, and Tween® 20 were obtained from Tianjin Damao Chemical Reagent Co., Ltd. (Tianjin, China). All other reagents were of analytical grade and were obtained from a local chemical supplier (Yunhui Trade Co., Ltd., Guangzhou, China).

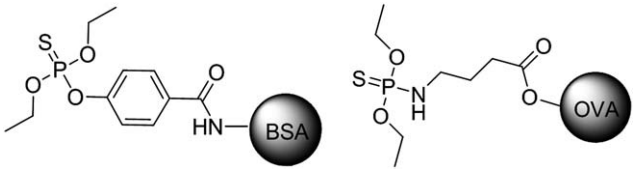

Fig. 1 Immunogen (hapten 1-BSA) and coating antigen (hapten 2-OVA) used in the immunoassay.

The ciELISA was carried out in 96-well polystyrene microplates (Xiamen Yunpeng Technology Development Co., Ltd, Xiamen, China). ciELISA plates were washed using a Multiskan MK2 microplate washer (Thermo Scientific, Hudson, NH, USA). Absorbance was measured at a wavelength of 450 nm using a Multiskan MK3 microplate reader (Thermo Scientific, Hudson, NH, USA).

2.2 ciELISA optimization

The ciELISA conditions such as the concentration of coating antigen, the dilution of antibody and HRP-IgG, and the incubation time and temperature were pre-optimized. Criteria used to evaluate immunoassay performance were maximal absorbance (A_{max}) and IC_{50} values. A_{max}/IC_{50} is a convenient estimate of an influence on ELISA sensitivity, a higher-ratio indicates higher-sensitivity.[19] The evaluation of ionic strength, pH, organic solvent and Tween® 20 concentrations on immunoassay behavior were determined in this study.

Effect of ionic strength. The effect of ionic strength on ciELISA performance was evaluated using different concentrations of PBS to dilute the parathion standard and antibody. The different PBS concentrations were prepared by diluting 10 × PBS (0.1 mol L^{-1}, pH 7.4) with distilled water. The pH value of the prepared PBS solutions was kept at 7.4 to eliminate the influence of pH.

Effect of pH. The effect of pH on ciELISA performance was studied using 2 × PBS (0.02 mol L^{-1}) to dilute the parathion standard and antibody over the pH range of 5.4 to 9.4. The PBS solutions with different pH values were prepared by changing the amounts of Na_2HPO_4 and KH_2PO_4; whereas, the concentration of NaCl and KCl remained the same to eliminate the influence of ionic strength.

Effect of Tween® 20 concentration. The effect of Tween® 20 on ciELISA performance was evaluated by diluting the parathion standard and antibody with 2 × PBS (pH 6.2) containing different concentrations of Tween® 20 (from 0 to 0.5%).

Effect of organic solvents. The effect of organic solvents on ciELISA performance was evaluated by using 2 × PBS (pH 6.2) containing different solvent concentrations (from 0 to 10%) of

methanol, acetone, and DMF to dilute the parathion standard and antibody.

2.3 Optimized ciELISA protocol

ciELISA plates were coated with the coating antigen (20 ng mL^{-1}, 100 μL $well^{-1}$) in carbonate buffer (0.05 mol L^{-1}, pH 9.6) overnight at 4 °C. The wells were washed 5 times with PBST (0.01 mol L^{-1} phosphate buffered saline (PBS) containing 0.05% Tween® 20, pH 7.4), and blocked with 5% skim milk in PBS (200 μL $well^{-1}$) for 2 h. After washing 2 times with PBST, the plates were dried at 37 °C overnight. OP standards or samples (50 μL $well^{-1}$) were diluted with 2 × PBS (0.02 mol L^{-1}, pH 6.2) containing 5% methanol and added to the wells followed by addition of the antibody (50 μL $well^{-1}$) diluted with 2 × PBS (0.02 mol L^{-1}, pH 6.2). After incubation for 40 min at 37 °C, the plates were washed 5 times with PBST. HRP-IgG diluted 1 : 4000 in PBST (100 μL $well^{-1}$) was then added to the wells. Following another 40 min incubation at 37 °C the plates were washed 5 times with the PBST solution, and the TMB solution (100 μL $well^{-1}$) was added to the wells and incubated for 10 min at 37 °C. The reaction was stopped by addition of 2M H_2SO_4 (50 μL $well^{-1}$), and the absorbance was recorded at 450 nm. Competitive curves were obtained by plotting absorbance against the logarithm of analyte concentration. The sigmoid curves were generated by using OriginPro 7.5 software (OriginLab Corp., Northampton, MA, USA). The IC_{50} value was defined as the concentration of analyte that produces a 50% decrease of the maximum normalized response. The limit of detection (LOD) was defined as the concentration of analyte that produces a 10% of the maximum normalized response (IC_{10}). The limit of quantification (LOQ) was defined as the lower and upper limits of quantification, which refers to the IC_{20}–IC_{80} linear range.[20]

2.4 Cross-reactivity study

The specificity of the ciELISA was determined using eighteen *O*,*O*-diethyl OPs and thirteen *O*,*O*-dimethyl OPs under optimized conditions. The percent cross-reactivity (CR) values were calculated according to the following equation:[21]

$$CR(\%) = [IC_{50}\ (\text{hapten 1, } \mu\text{mol } L^{-1})/IC_{50}\ (\text{cross-reactant, } \mu\text{mol } L^{-1})] \times 100.$$

2.5 Environmental water sample analysis

Pond water samples were collected on the campus of South China Agricultural University (SCAU, Guangzhou, China). River water samples were collected from the Zhujiang River, the largest drinking water source for the city of Guangzhou, China. Paddy water samples were collected in agricultural areas near SCAU. The pH value of the water samples was also measured. All water samples were filtered over a mixed cellulose ester microporous membrane (Shanghai Xingya Purification Material Factory, Shanghai, China) to remove particles larger than 0.45 μm, and the samples were then stored at 4 °C until further use. Prior to ciELISA analysis, all water samples were confirmed to be OP-free by high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) analysis, which was completed by the China National Analytical Center, Guangzhou, China.

Matrix effects. The parathion standard was diluted with environmental water samples to obtain dose dependent standard curves to evaluate if the water samples had a matrix effect on the ciELISA performance. These standard curves were then compared to a standard curve of parathion prepared in 2 × PBS (pH 6.2). Water samples were also diluted in both 2 × PBS (pH 6.2) and 4 × PBS (pH 6.2) to investigate ways to eliminate the matrix effect.

Recovery test. The water samples were spiked with different concentrations of parathion, phoxim, and triazophos dissolved in methanol. The concentration of spiked OPs covered the quantitative working range for each OP. The final concentration of methanol was kept to 5%. Prior to ciELISA analysis, the spiked samples were diluted (1 : 1 v/v) with 4× PBS (pH 6.2). The diluted spiked water samples (50 μL) were used for ciELISA analysis without further pretreatment.

Blind sample test. Ten water samples were randomly spiked with different OPs at different concentrations and serially numbered. Only the person who prepared the samples knew the details of the ten blind samples. The blind samples were then analyzed with the developed ciELISA by a different person. Parathion was used to develop the control standard curve. The average absorbance of each sample was recorded and then used to calculate the percent inhibition using the following equation: $I(\%) = [(A_0–A_x)/A_0] \times 100$, where A_0 is the absorbance of the control (2 × PBS) and A_x is the absorbance of the blind samples at 450 nm. The samples that demonstrated an inhibition lower than 10% were regarded as negative samples, and samples with a percent inhibition higher than 10% were considered positive.

HPLC-MS/MS analysis. HPLC-MS/MS was used for simultaneous determination of coumaphos, parathion, phoxim, quinalphos, triazophos, dichlofenthion, azinphos-ethyl, phosalone, isochlorthion, parathion-methyl, cyanophos, disulfoton and phorate was developed according to the National Standard methods (GB/T 23214-2008). The 1200 series HPLC system (Agilent Technologies, Santa Clara, CA, USA) was used for separation of the studied pesticides on a Hypersil BDS C8 column (100 mm × 2.1 mm i.d., 2.4 μm particle size; Thermo Scientific, Hudson, NH, USA). Mobile phase A consisted of 0.2% acetic acid and 10 mmol L^{-1} ammonium acetate in water, mobile phase B consisted of 0.2% acetic acid in acetonitrile, and they were used in the following gradient profile: 0 min, 55% A and 45% B; 8 min, 10% A and 90% B; then 8.1–14 min 55% A and 45% B. The flow rate of the mobile phase was 0.2 mL min^{-1} and an aliquot of 10 μL of each sample was injected into the HPLC system. The mass spectra were obtained with an Agilent 6410 Triple Quad mass spectrometer (Agilent Technologies, Lexington, MA, USA) using the electrospray ionization (ESI) technique. All pesticides were analyzed in the positive ionization (PI) mode.

For water sample analysis, 10 mL of each water sample were mixed with 20 mL of acetonitrile (containing 1% acetic acid) for 2

min. Anhydrous sodium acetate (2 g) was added to the mixture and vortexed for 1 min. Anhydrous magnesium sulfate (7.5 g) was added and the mixture was shaken for 5 min. The mixture was centrifuged at 5000 × *g* for 5 min. The supernatant (10 mL) was transferred and evaporated to about 1 mL at 40 °C under vacuum, and then transferred onto a Sep-Pak Vac column (preconditioned with 5 mL of acetonitrile/methylbenzene (3 : 1 v/v), Waters, Germany). The column was washed with 25 mL of acetonitrile/methylbenzene (3 : 1 v/v) and the eluent was evaporated to dryness under vacuum. A 1 mL mixture of acetonitrile/water (3 : 2 v/v) was used to dissolve the residue and the samples were submitted for HPLC-MS/MS analysis.

3. Results and discussion

3.1 ciELISA optimization

It is well known that immunoassay performance may be affected by many physicochemical features of the media and by a variety of experimental conditions.[20] The sensitivity of a competitive ELISA is influenced by the amounts of both the primary antibody and the coating antigen. In a previous study,[18] the concentration of coating antigen used was 30 ng mL^{-1}, while the dilution of antibody and HRP-IgG was 1 : 64 000 and 1 : 6000, respectively. In this study, the concentration of coating antigen was reduced to 20 ng mL^{-1}. The resulting decrease of absorbance was offset by changing the dilution of antibody and HRP-IgG to 1 : 32000 and 1 : 4000, respectively. A small improvement in assay sensitivity was obtained (IC_{50} for parathion was improved from 4.21 ± 0.15 ng mL^{-1} to 3.12 ± 0.11 ng mL^{-1}, $n = 3$) as a result of an improvement in the competitive ability of the analytes. Results for incubation temperature and time demonstrated that the higher the temperature, the shorter the amount of time was needed for incubation. To develop a fast screening method, a decrease in incubation time is very important. Therefore, the temperature chosen for the best performance of the ciELISA was 37 °C. However, performing an ELISA at elevated temperatures may enhance non-specific binding.[22] But the standard curves obtained at 37 °C in this study showed a low background, which indicated no non-specific binding was found in the test. Increased antibody incubation time resulted in higher maximum absorbance, but showed no significant influence on assay sensitivity when the time increased above 40 min. Therefore, a 40 min incubation of antibody, a 40 min incubation of HRP-IgG, and a 10 min color development were chosen as the final optimum incubation times, and all incubations were conducted at 37 °C. These conditions were used throughout this work.

Effect of ionic strength. Because antigen–antibody binding is characterized by weak intermolecular bonds, a change in the ionic strength could affect the interactions.[23] The effect of ionic strength on assay performance is shown in Fig. 2A. The results show that with increased ionic strength, the A_{max} value gradually decreased and then slightly increased when the ionic strength achieved 10 × PBS. The lowest IC_{50} was obtained at a concentration of 2 × PBS, which is also the concentration resulting in the highest A_{max}/IC_{50} value. Therefore, 2 × PBS was chosen as the optimum ionic strength. Although the influence of salt concentration on the performance of pesticide immunoassays varies, 1–2 × PBS was the optimal buffer concentration for many pesticide immunoassays.[24–27] In addition, the effect of salt concentration on ELISA performance appears to be different with different ELISA formats. Compared to the ciELISA format (immobilized conjugate), the effect of salt concentration was not the same in the antibody competitive direct ELISA (cdELISA) format (immobilized antibody).[24,26,28,29] The recognition of the conjugated hapten diminished or increased in the ciELISA or cdELISA, respectively, when the salt concentration increased.[29] But overall, it was suggested that the interaction between antibodies and hydrophobic analytes was favored in polar environments.[30]

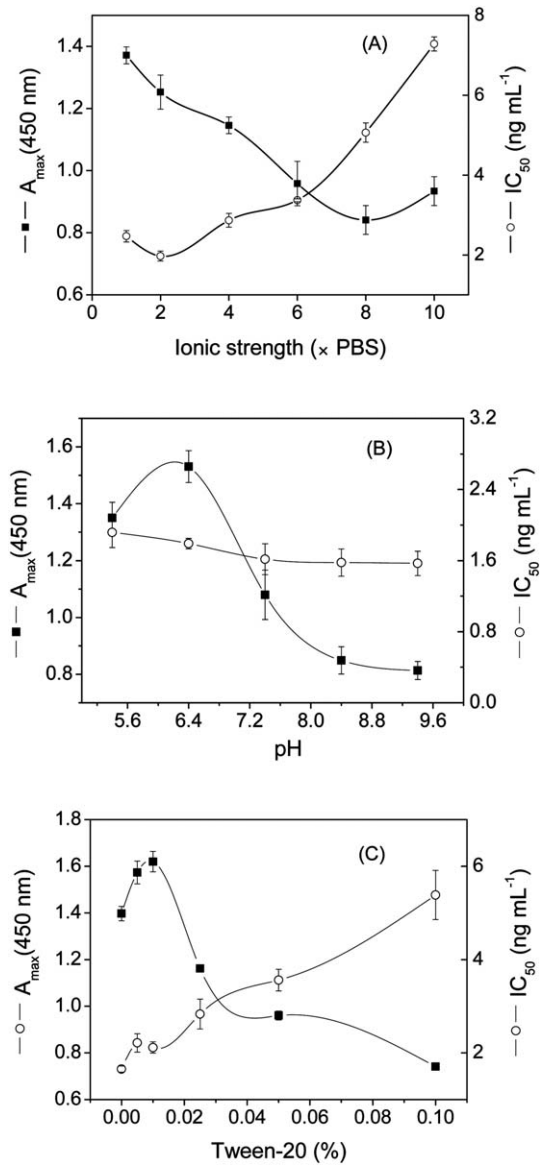

Fig. 2 Influence of ionic strength (A), pH (B), and concentration of Tween® 20 (C) on the ciELISA of parathion. Each point represents the average of three replicates and the standard deviation of the mean.

Effect of pH. The change of pH can also affect antigen–antibody interactions.[24] Fig. 2B presents the effects of pH on assay

performance. The pH significantly influenced the A_{max} value, which first increased and then decreased with pH values ranging from 5.4 to 9.4. However, the sensitivity was quite stable with the changing pH values. Since the A_{max}/IC_{50} ratio was maximal between pH 6.0 to 6.4, and most *O*,*O*-diethyl OPs are more stable in acidic rather than in neutral environments,[11,31,32] pH 6.2 appeared to be a reasonable choice for the competition buffer. Moreover, the pH values of collected environmental water samples ranged from 5.5 to 6.5. Therefore, selection of a buffer with a pH of around 6 may help reduce the matrix effect. Most studies indicate that acidic or neutral environments are reasonable choices for the competition step of pesticide immunoassays.[24–28]

Effect of Tween® 20 concentration. Tween® 20 is a nonionic surfactant commonly used in immunoassay protocols to reduce non-specific interactions.[33] Using the established pH and buffer concentration, the effect of the surfactant Tween® 20 concentration on the signal and sensitivity was studied. The results are presented in Fig. 2C. The A_{max} value increased when the concentration of Tween® 20 was lower than 0.01% and then markedly decreased. Although the sensitivity was observed to be increased slightly when 0.01% Tween® 20 was used (highest A_{max}), the maximum A_{max}/IC_{50} ratio was obtained when no Tween® 20 was added. Several studies also demonstrated that the presence of Tween® 20 was detrimental to the sensitivity of pesticide immunoassays.[24–30] Galve *et al.* indicated that highly-polar compounds such as the chlorophenols may not be affected by Tween® 20 in regard to nonspecific interactions.[34] On the other hand, it was also demonstrated in several works that a lower concentration of Tween® 20 can improve the immunoassay detectability for small non-polar organic analytes such as chlorpyrifos and triclosan.[35,36] Since the presence of Tween® 20 did not improve the assay sensitivity significantly, the buffer used for the optimized assay contained no Tween® 20.

Tolerance of organic solvent. Most OPs are not readily soluble in water; therefore, a polar organic solvent is often added to the assay buffer of OP ELISAs.[28] The effects of different percentages of methanol, acetone, and DMF on ciELISA performance were studied here. As seen in Fig. 3, the A_{max} and IC_{50} values increased compared to the control as the content of methanol increased. However, the A_{max}/IC_{50} value was not significantly changed when the concentration of methanol was below 2.5%. In contrast, the absorbance decreased with increasing solvent concentrations of acetone and DMF. The addition of acetone and DMF also significantly decreased the assay sensitivity. Similarly, other research groups reported that methanol caused the least negative effects on pesticide immunoassays.[28,35] Considering the poor water solubility of some OPs, such as dichlofenthion and phosalon, organic solvents are required during extraction of samples; therefore, 2.5% methanol was added to the assay buffer.

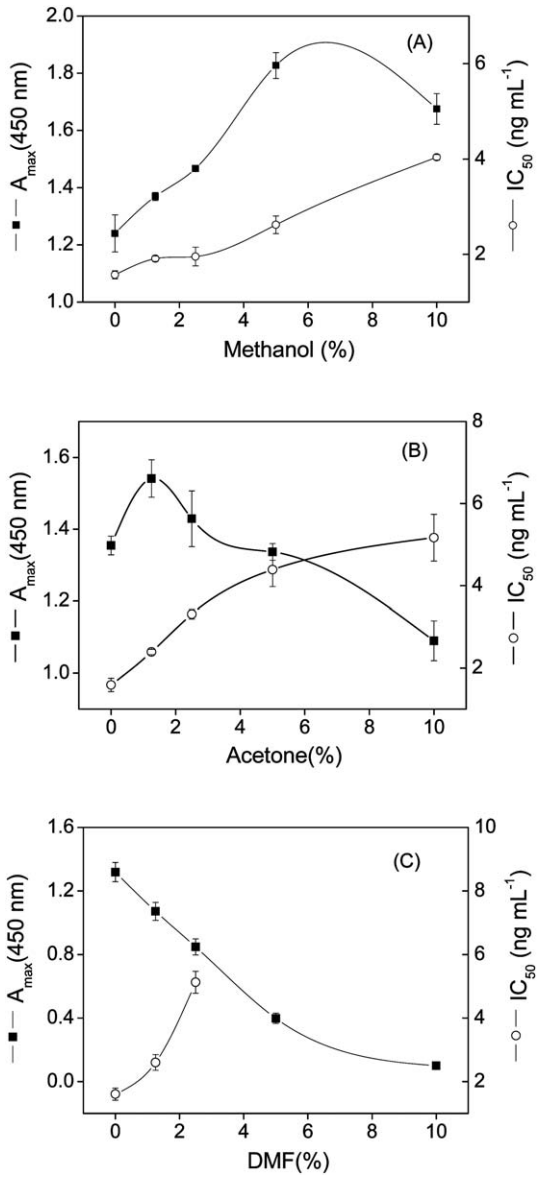

Fig. 3 Influence of the organic solvents, methanol (A), acetone (B), and DMF (C) on the ciELISA of parathion. Each point represents the average of three replicates and the standard deviation of the mean.

3.2 Cross-reactivity studies

Eighteen *O*,*O*-diethyl OPs and thirteen *O*,*O*-dimethyl OPs were used to study the specificity of the developed immunoassay. As shown in Table 1, the antibody demonstrated higher cross-reactivity (CR) for *O*,*O*-diethyl OPs than for *O*,*O*-dimethyl OPs. Twelve OPs had a CR higher than 10% and six of them were higher than 100%. In contrast to the previous results,[18] the sensitivity of the ciELISA in this study showed a three to five-fold improvement for most OPs following optimization. After ciELISA optimization, nine OPs could be detected at a concentration level that was less than 10 ng mL^{-1}, and four other OPs could be detected at levels that were just under 30 ng mL^{-1} (Table 2).

Environmental water is the primary source of drinking water. In many developed countries, maximum residue limits (MRLs) have been established for pesticides in drinking water. The U.S. Environmental Protection Agency (EPA) Method 525 has a maximum allowable risk level for OPs in drinking water ranging from 1 to 25 ng mL^{-1}. In the European Union (EU),

Table 1 Cross-reactivity of the antibody for *O*,*O*-diethyl and *O*,*O*-dimethyl OPs

No.	Analytes	IC_{50}[a]	CR(%)[b]	No.	Analytes	IC_{50}	CR(%)
1	Coumaphos	0.19	18621.0	17	Chlorpyrifos	1131.6	3.0
2	Parathion	1.53	1856.6	18	Fenitrothion[c]	1133.8	2.4
3	Phoxim	7.82	372.0	19	Isazophos	1382.2	2.2
4	Quinalphos	15.17	191.8	20	Diazinon	1702.1	1.7
5	Dichlofenthion	20.56	149.5	21	Methacrifos[c]	2376.9	1.0
6	Triazophos	29.14	104.9	22	Pirimiphos-ethyl	3545.2	0.9
7	Hapten 1	28.31	100	23	Terbufos	>10 000	<0.3
8	Azinphos-ethyl	38.35	87.8	24	Ethion	>10 000	<0.4
9	Phosalone	67.67	53.0	25	Famphur[c]	>10 000	<0.3
10	Isochlorthion[c]	87.19	33.3	26	Fenthion[c]	>10 000	<0.3
11	Parathion-methyl[c]	197.96	13.0	27	Chlorpyrifos-ethyl[c]	>10 000	<0.3
12	Cyanophos[c]	213.06	11.1	28	Bromophos[c]	>10 000	<0.4
13	Disulfoton	246.29	10.1	29	Temephos[c]	>10 000	<0.5
14	Phorate	265.20	9.4	30	Fenchlorphos[c]	>10 000	<0.3
15	Bromophos-ethyl	620.40	6.2	31	Phenthoate[c]	>10 000	<0.3
16	Sulfotep	984.13	3.2	32	Lodofenphos[c]	>10 000	<0.4

[a] IC_{50} values are in units of ng mL^{-1}. [b] Percent CR was calculated by the equation (IC_{50} of hapten 1/IC_{50} of cross-reactant) × 100 using units of moles (μmol L^{-1}) for IC_{50}. [c] *O*,*O*-Dimethyl OPs.

Table 2 OPs that can be detected at a concentration below 30 ng mL^{-1}

Analytes	LOD[a]	LOQ[b]
1 Coumaphos	0.02	0.04–0.85
2 Parathion	0.17	0.38–6.76
3 Phoxim	1.07	2.22–30.08
4 Quinalphos	2.13	4.33–86.19
5 Triazophos	2.88	6.85–130.13
6 Dichlofenthion	3.68	6.82–72.9
7 Azinphos-ethyl	4.02	10.17–180.28
8 Phosalone	8.43	17.94–301.42
9 Isochlorthion	9.12	20.55–417.31
10 Parathion-methyl	21.28	47.35–984.34
11 Cyanophos	23.91	53.77–1175.65
12 Disulfoton	24.16	56.15–1180.26
13 Phorate	27.38	68.72–959.86

[a] LOD is the limit of detection (IC_{10}). [b] LOQ is the lower (IC_{20}) and upper (IC_{80}) limit of quantification.

a maximum allowable concentration of 0.1 ng mL^{-1} for each individual pesticide in drinking water is in force (EU Directives, 1980 and 1998). However, in many developing countries, such as China, no MRLs for OPs in environmental water and drinking water have been established. The MRLs for foodstuffs (such as rice, vegetables and fruits) are in the range from 10 to 2000 ng mL^{-1} in China according to the Chinese National Standard (GB 2763-2005 and GB26130-2010). Due to the large scale use of pesticides in China, multi-residues of pesticides in foodstuffs and environmental water is a serious problem. Twelve pesticides, including parathion and parathion-methyl, were detected in Taihu Lake, one of the five largest lakes in China.[37] Multi-residues of pesticides were also found in foodstuffs[38,39] and soil[40] at high-levels. To avoid adverse impact on public health, it is important to develop analytical methods with high-sensitivity and low-cost for screening multi-residues of pesticides. The limit of detections (LODs) of current used OPs screening method in China (cholinesterase-based spectrophotometric methods, GB/T 5009.199-2003) are at the level of μg mL^{-1} (*e.g.* 1.0 μg mL^{-1} for parathion and 0.3 μg mL^{-1} for phoxim). Compared with this method, the proposed ciELISA made a significant improvement in the sensitivity. It demonstrated that the developed ciELISA may be useful as a screening tool for OP residues, and this method may be especially useful in Asian countries where OPs are extensively used.

3.3 Environmental water sample analysis

Matrix effects. One of the major advantages of using an immunoassay is that the samples can be analyzed without complex pretreatment.[19] Therefore, it is important to know whether the calibration curves constructed with standard solutions can be used with real samples. To evaluate the environmental water sample matrix effect on immunoassay performance, parathion standards were diluted in both environmental water samples and in 2 × PBS to develop calibration curves. As shown in Fig. 4, both the A_{max} and IC_{50} slightly increased when parathion was diluted with environmental water samples (*e.g.*, pond water). Our studies revealed that the ionic strength and pH value of the media significantly influenced immunoassay performance. To eliminate matrix effects, samples

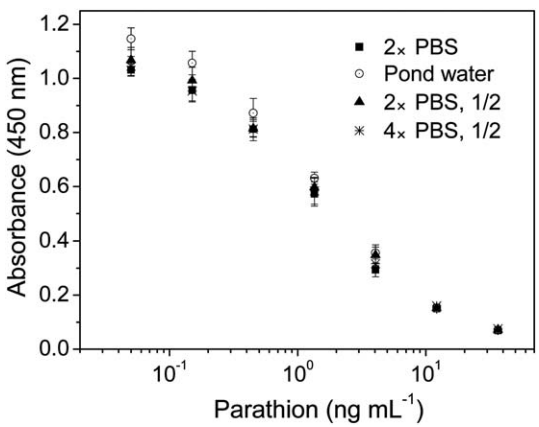

Fig. 4 Matrix effect of pond water on the parathion ciELISA.

may be diluted in assay buffer.[20] We used 2 × PBS (pH 6.2) and 4 × PBS (pH 6.2) to dilute water samples to determine if dilution could eliminate the matrix effect. It was determined that making a sample dilution of water/2 × PBS (1 : 3 v/v) or water/4 × PBS (1 : 1 v/v) would completely eliminate the matrix effect. However, a large dilution factor may result in causing the OP concentration to move out of the quantitative working range of the developed immunoassay when analyzing real water samples. Therefore, water samples were diluted as follows: water sample/4 × PBS (1 : 1 v/v).

Recovery test. Pond water, river water, and paddy water samples with a proven absence of OPs were spiked at different concentrations with coumaphos, disulfoton, parathion, phosalone, phoxim, and triazophos, and were analyzed five times on the same plate by the developed ciELISA. Selected dose–response curves and calibration curves in the working range for parathion, phoxim, and triazophos are presented in Fig. 5. The results of recovery tests are shown in Table 3. The average recovery and coefficient of variation (CV) was 95.2% and 14.3%, respectively, and the recovery ranged from 60.0% to 120.6% resulting in CVs from 6.1% to 22.3%, respectively. The findings indicated that the reproducibility of the ciELISA determination was satisfactory.

Blind sample test. The quantitative analysis of individual OPs is not possible using a broad-specificity immunoassay because the antibody has affinity to many different OPs.[41] Most reported broad-specificity immunoassays have failed to describe the determination of analytes in blind samples.[15–17,18] However, it is feasible to develop a broad-specificity immunoassay as a semi-quantitative screening method. In this study, "percent inhibition rate" was used as a parameter to describe whether a sample contained one or a number of OPs, or whether it did not contain OPs. When the samples showed a percent inhibition lower than 10%, they were regarded as negative, and they were considered positive when the percent inhibition was higher than 10%. The higher the percent inhibition was, the more positive the sample. The developed method would be advantageous to screen out samples that did not contain OPs, and the positive samples could then be further analyzed by instrumental methods.

Ten water samples with unknown amounts of OPs were analyzed in a blind study with the developed ciELISA. The negative control was 2 × PBS (pH 6.2). The results are shown in Table 4. Five negative samples were found during the screening test. Among them, four samples were free of OPs, and the last sample (number 6) was spiked with 0.5 ng mL^{-1} of phoxim, 1 ng mL^{-1} of quinalphos and 5 ng mL^{-1} of phosalone. Each of these OPs was spiked at a level below the LOD for the individual OP. The results indicated that there was not a direct additive effect when a sample contained two or more OPs. The results of sample 2 (5 ng mL^{-1} of azinphos-ethyl), sample 3 (30 ng mL^{-1} of parathion-methyl), and sample 5 (5 ng mL^{-1} of azinphos-ethyl and 30 ng mL^{-1} of parathion-methyl) re-confirmed this conclusion. The percent inhibition of sample 5 was only slightly greater than either sample 2 or sample 3.

HPLC-MS/MS analysis. Although the broad-specificity ciELISA was able to identify the positive samples from negative samples, it was unable to determine which OP(s) were present and the levels of those OP(s). To solve this problem, a HPLC-MS/MS method for multi-analyte analysis of 13 OPs was developed that could detect low levels of the OPs. The limit of quantification (LOQ) of HPLC-MS/MS for 13 OPs in water samples was as follows (ng mL^{-1}): coumaphos (0.5), parathion (0.5), phoxim (0.2), quinalphos (0.2), triazophos (0.2), dichlofenthion (1.0), azinphos-ethyl (0.2), phosalone (0.2),

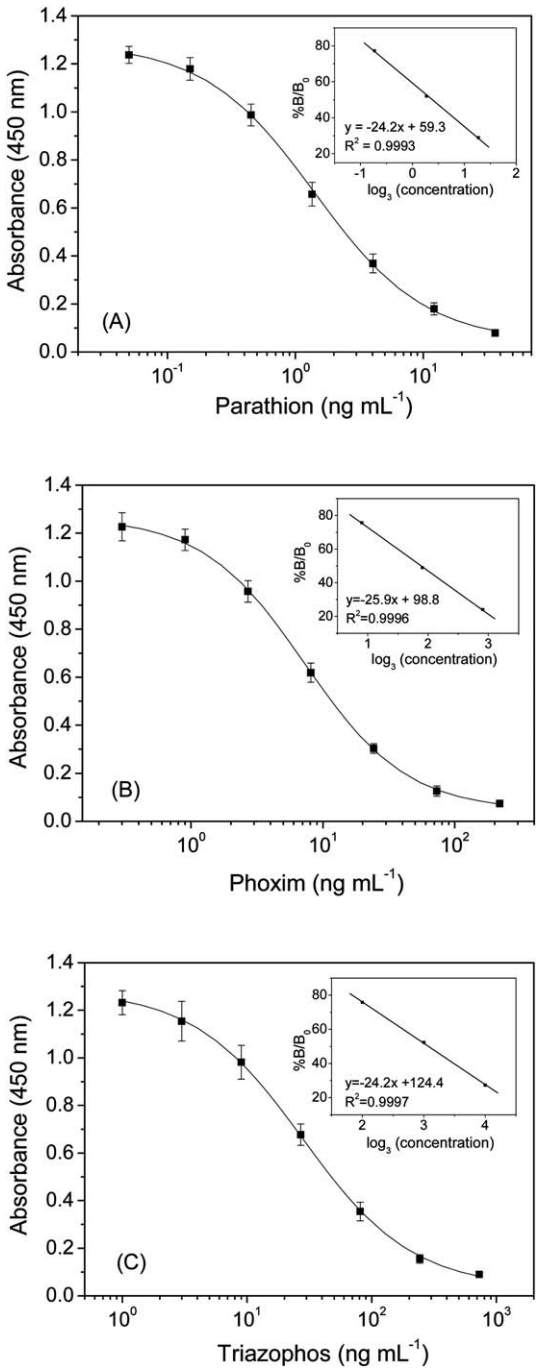

Fig. 5 Dose–response curves and calibration curves in the working range (insert) for the three selected OPs, parathion (A), phoxim (B), and triazophos (C). Each point represents the average of five replicates and the standard deviation of the mean.

Table 3 Recovery of three selected OPs from three spiked water samples ($n = 5$)[a]

		Pond water			River water			Paddy water		
Analytes	Added	Found	R(%)	CV(%)	Found	R(%)	CV(%)	Found	R(%)	CV(%)
Coumaphos	0.05	0.03	60.0	15.2	0.04	80.0	17.2	0.04	80.0	12.6
	0.15	0.17	113.3	14.1	0.18	120.0	10.3	0.14	93.3	16.9
	0.45	0.52	115.6	9.7	0.37	82.8	14.0	0.39	86.7	20.1
Parathion	0.45	0.40	89.5	12.6	0.49	108.1	10.5	0.41	91.8	19.6
	1.35	1.26	93.3	11.6	1.20	88.9	11.3	1.44	106.5	15.3
	4.05	4.10	101.2	18.3	4.10	101.3	15.5	0.30	94.4	13.2
Phoxim	2.7	2.8	103.3	11.7	2.5	92.5	14.7	2.9	106.5	15.3
	8.1	7.0	86.3	13.8	8.3	102.0	20.2	7.34	90.6	11.4
	24.3	21.5	88.4	9.5	22.2	91.5	12.3	19.8	81.5	6.1
Triazophos	9.0	8.5	94.1	11.9	10.4	115.1	14.5	7.8	87.1	16.4
	27.0	29.3	108.5	15.4	27.4	101.3	10.5	31.0	115.0	20.9
	81.0	74.3	91.7	8.3	68.8	85.0	12.3	77.3	95.4	12.2
Phosalone	25.0	17.9	71.6	21.2	22.3	89.2	7.8	27.9	111.6	14.7
	75.0	88.2	117.6	17.8	71.4	95.2	19.6	79.5	106.0	16.3
	225.0	271.3	120.6	14.2	189.5	84.2	12.3	165.3	73.4	12.4
Disulfoton	80.0	52.7	65.9	22.3	81.2	101.5	14.5	71.4	89.3	17.2
	240.0	241.1	100.5	10.2	271.5	113.2	9.7	217.0	90.4	19.3
	720.0	625.7	86.9	17.1	693.0	96.3	17.2	614.8	85.4	13.5

[a] The amounts analytes added and found are in units of ng mL^{-1}. R is the percent recovery. CV is the coefficient of variation, and the data was obtained from five determinations performed on the same ELISA plate.

Table 4 Results of ten blind water samples by the developed ciELISA ($n = 5$)

Samples	A_{450nm}	Inhibition(%)[a]	Results[b]	HPLC-MS/MS[c]/ng mL^{-1}	Analytes added[c]/ng mL^{-1}
Control[d]	1.28 ± 0.03	0	–		None
1	1.26 ± 0.04	1.75	–	ND[e]	None
2	1.13 ± 0.03	11.27	+	Azi (2.33)	Azi[c] (5)
3	1.09 ± 0.02	15.02	+	Par-M (24.0)	Par-M[c] (30)
4	1.29 ± 0.03	−0.83	–	ND	None
5	1.04 ± 0.04	18.78	+	Azi (2.83)	Azi (5)
				Par-M (19.8)	Par-M (30)
6	1.21 ± 0.03	5.66	–	Phox (0.37)	Phox[c] (0.5)
				Qui (1.18)	Qui[c] (1)
				Phos (4.91)	Phos[d] (5)
7	0.89 ± 0.05	30.67	++	Tri (3.63)	Dic[c] (5)
				Azi (5.61)	Tri[c] (5)
					Azi (5)
8	0.79 ± 0.06	37.87	++	ND	Cou[c] (0.1)
9	1.33 ± 0.04	−3.99	–	ND	None
10	1.27 ± 0.08	0.73	–	ND	None

[a] Percent inhibition was calculated using the equation $[(A_0 - A_x)/A_0] \times 100$, where A_0 is the absorbance of the control at 450 nm, A_x is the absorbance of the samples. [b] +++ strong positive; ++ medium positive; + weak positive; – negative. [c] Azi = azinphos-ethyl, Par-M = parathion-methyl, Dic = dichlofenthion, Tri = triazophos, Phos = phosalone, Phox = phoxim, Qui = quinalphos, Cou = coumaphos. The LOQ was 0.2 ng mL^{-1} for azinphos-ethyl; 0.5 ng mL^{-1} for parathion-methyl; 0.2 ng mL^{-1} for phoxim; 0.2 ng mL^{-1} for quinalphos; 1.0 ng mL^{-1} for dichlofenthion; 0.2 ng mL^{-1} for triazophos; and 0.5 ng mL^{-1} for coumaphos. [d] The control used was 2 × PBS containing 2.5% methanol. [e] ND = Not detected (out of the LOQ).

isochlorthion (2), parathion-methyl (0.5), cyanophos (10), disulfoton (2.0) and phorate (1.0). The ten blind samples were then submitted for HPLC-MS/MS analysis and the results are presented in Table 4. All samples that were determined positive for OPs by screening with the ciELISA were also demonstrated to contain OPs by the HPLC-MS/MS method except for sample 8, which was spiked with a low level of coumaphos. This level was below the lower detection level of the HPLC-MS/MS method. The comparison of the two studies demonstrated that the results of the developed ciELISA were consistent with that of the HPLC-MS/MS determination.

4. Conclusions

In summary, a fast and sensitive broad-specificity ciELISA based on a previously obtained monoclonal antibody was optimized and applied to screen OP residues in environmental water samples. The optimal conditions for the assay are as follows: the coating antigen concentration was 20 ng mL^{-1}; antibody was diluted 1 : 32 000 with PBS (0.02 mol L^{-1}, pH 6.2) and competed against the target analyte dissolved in PBS (0.02 mol L^{-1}, pH 6.2) containing 5% methanol. Following optimization, the ciELISA showed a LOD ranging from 0.017 to 30 ng mL^{-1} for thirteen

OPs. The accuracy and reproducibility of the ciELISA determination was acceptable as was determined by spiked environmental water samples. Although the ciELISA was unable to distinguish which OPs were present in the water samples and was unable to provide a quantitative analysis, it was ideally suited as a semi-quantitative screening test capable of analyzing a large number of samples that could be screened to remove all OP negative samples prior to chromatographic analysis. The broad-specificity ELISA kit has been constructed and will be commercially available in the near future.

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